

Development and Validation of a Simple and Robust Bioanalytical Method to the Quantification of Tafamidis in Human Plasma using LC-MS/MS

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ABSTRACT

Objectives: Amyloid diseases are a rare class of disorders that have gained significant attention for therapeutic advancements. Among the key proteins implicated, transthyretin plays a central role in the progression and clinical symptoms of these diseases. Tafamidis, an approved therapeutic agent, has demonstrated considerable efficacy in mitigating symptoms associated with transthyretin-related amyloid conditions. Despite its clinical significance, robust bioanalytical methodologies for the accurate quantification of Tafamidis in biological matrices remain limited. **Materials and Methods:** This study focuses on the development and validation of a precise bioanalytical method to quantify Tafamidis in human plasma using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The method was calibrated over a range of 5-4000 ng/mL, with chromatographic separation performed using an isocratic mobile phase on a Gemini NX C18 column. Mass transitions were optimized at 307.400 → 262.400 for Tafamidis and 313.900 → 270.000 for Tafamidis-¹³C₆. A liquid-liquid extraction approach ensured efficient recovery of Tafamidis from plasma. **Results and Discussion:** Validated per USFDA regulatory guidelines, the method demonstrated excellent precision, accuracy, and stability, with correlation coefficients exceeding 0.99 and recovery rates reaching 70%. Stability was confirmed for up to 10 days in matrix and aqueous samples. **Conclusion:** This robust, validated method offers high reliability and suitability for analytical applications and quantitative clinical investigations involving Tafamidis.

Keywords: Amyloid Diseases, Transthyretin, Protein, Method development, Method validation.

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Received: 21-05-2026;

Revised: 04-06-2026;

Accepted: 17-08-2026.

INTRODUCTION

Transthyretin (TTR) is a critical protein in the human body responsible for transporting thyroxine (T₄) and retinol via its binding capacity in the bloodstream and Cerebrospinal Fluid (CSF). By stabilizing T₄ at its binding sites, TTR prevents its dissociation, misfolding, and aggregation, thereby maintaining essential physiological functions.¹ However, destabilization and misfolding of TTR can result in Amyloid Diseases (ADs) such as Senile Systemic Amyloidosis (SSM), Familial Amyloid

Polyneuropathy (FAP), and Familial Amyloid Cardiomyopathy (FAC), all of which are potentially fatal.^{2,3} These diseases may arise from hereditary or non-hereditary factors, with chromosomal mutations often implicated in the misfolding of TTR. Misfolded TTR accumulates in tissues, leading to severe consequences: in SSM and FAC, it causes cardiac stiffness, wall thickening, and eventual heart dysfunction, while in FAP, amyloid deposition weakens muscles and disrupts neuronal function, culminating in polyneuropathy. Furthermore, Amyloidosis Diseases (ALDs) are not limited to TTR-related conditions but may also stem from the misfolding of other proteins, highlighting the heterogeneity of these disorders.

Globally, the prevalence of ALDs is increasing, and no permanent cure currently exists. However, recent clinical Trials on Tafamidis (TFM) and diflunisal have shown promise in controlling the progression of TTR-related amyloidosis symptoms.⁴ Tafamidis,



DOI: 10.5530/ijper.20262396

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a pharmacoperone, acts as a stabilizer by preserving the native tetrameric structure of TTR, thereby preventing its dissociation into monomers, a key step in amyloid formation. This stabilization mitigates symptoms such as neuropathy and cardiac dysfunction. Recognized as a breakthrough, TFM is a first-in-class medication and an orphan drug approved by the US FDA for the treatment of TTR amyloidosis.⁵ Although discovered in 2003, its clinical use began in 2011 in the European Union, followed by Japan and the United States.⁶ Given the rise of emerging diseases and the need to manage rare conditions like ALDs, the demand for TFM and similar compounds has grown significantly. The ongoing global research efforts to develop novel therapies for rare diseases, including ALDs, and emerging challenges such as cancer, COVID-19, and antibiotic resistance, hold immense promise. These studies not only contribute to the development of innovative drugs but also enhance our understanding of pharmacokinetics and pharmacodynamics, paving the way for more effective treatments in the future.⁷⁻¹¹

Analytical methods are fundamental to the development and evaluation of novel and generic drug products, particularly bioanalytical techniques that support Bioavailability and Bioequivalence (BA/BE) studies during clinical development. Reliable quantification of drug concentrations in biological matrices is essential to ensure accurate pharmacokinetic and bioequivalence assessments in accordance with regulatory expectations.^{12,13} Despite the clinical importance of TFM, a transthyretin stabilizer used in the treatment of transthyretin amyloidosis, limited analytical methods have been reported for its qualitative and quantitative determination in human plasma. The available reported literature reveals notable limitations in existing methodologies. For example, Miglena Smerikarova *et al.*,¹⁴ reported an HPLC-UV method for TFM analysis. However, the method lacks sensitivity and is restricted to microgram-level detection, limiting its suitability for clinical pharmacokinetic or BA/BE studies. Hyun *et al.*,¹⁵ developed an LC-MS/MS method with a calibration range of 3-3000 ng/mL, but the method employed a structurally similar internal standard rather than an isotope-labelled internal standard, potentially compromising correction for matrix effects and extraction variability. In addition, the relatively narrow calibration range limits its applicability across different dosing regimens and concentration levels. The same method was later applied by Lee *et al.*, for pharmacokinetic evaluation of TFM, further highlighting the continued reliance on an approach with limited sensitivity and robustness.¹⁶

Furthermore, existing reports provide limited discussion on method robustness, extraction simplicity, stability performance, and analytical throughput, all of which are critical parameters for routine bioanalysis in regulated BA/BE studies. These gaps underscore the need for a more sensitive, selective, and regulatory-compliant bioanalytical method capable of reliable

quantification of TFM over a wider concentration range using a straightforward and reproducible extraction procedure.

In this context, the present study aims to develop and validate a simple, robust, and high-throughput LC-MS/MS method for the quantification of TFM in human plasma in accordance with USFDA bioanalytical method validation guidelines. The proposed method employs a straightforward sample preparation procedure, an isotope-labelled internal standard for improved accuracy, and a sensitive LC-MS/MS detection strategy to achieve reliable quantification across a broad calibration range. By addressing the limitations of previously reported methods, the developed method enhances analytical sensitivity, robustness, and operational efficiency, thereby providing a suitable tool for clinical pharmacokinetic, bioavailability, and bioequivalence studies.

MATERIALS AND METHODS

Materials

Chemicals and Instrumentation

The solvents utilized in the current research were of LC-MS grade and sourced from JT Baker, Avantor, USA. The drugs, Tafamidis (TFM, analyte) and tafamidis-13C6 (internal standard, IS), were procured from Vivan Life Sciences, Mumbai, India. Human plasma containing dipotassium Ethylenediaminetetraacetic Acid (K₂EDTA) was obtained from M/S Laxmi Sai Chemical Lab, Hyderabad, India. The Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) system, QTRAP 4500, was supplied by AB SCIEX, USA. The analytical column used was a Gemini NX-C18 column (100 × 4.6 mm, 5 µm) from Phenomenex, USA.

Preparation of Stock solutions

The analyte and Internal Standard (IS) were dissolved in a methanol:dimethyl sulfoxide solution (50:50, v/v) to achieve stock concentrations of 1 mg/mL for the analyte and 0.2 mg/mL for the IS. These stock solutions were subsequently diluted fivefold using a methanol:water solution (50:50, v/v) to prepare 2% spiking samples for use in biological matrices. The IS working solution was further diluted to a concentration of 500 ng/mL, which was consistently utilized throughout the validation process.

Preparation of Calibration Curve (CC) and Quality Control (QC) Samples

A Calibration Curve (CC) comprising ten non-zero points was established within the range of 5 ng/mL to 4000 ng/mL, corresponding to a Lower Limit of Quantification (LLOQ) of 5 ng/mL and an Upper Limit of Quantification (ULOQ) of 4000 ng/mL. The calibration points were as follows: 5, 10, 25, 75, 150, 300, 750, 1500, 3000, and 4000 ng/mL. Quality Control (QC) samples were prepared at various concentration levels: high-quality control (HQC, 2900 ng/mL), middle-quality control (MQC, 1600 ng/mL), lower-quality control (LQC, 15 ng/mL), lower limit

of Quantification Quality Control (LLOQ QC, 5 ng/mL), and Dilution Quality Control (DQC, 8000 ng/mL). The DQC samples were evaluated using a dilution factor of 5. The prepared CC and QC dilutions were used to spike plasma samples at a 2% spiking ratio. The spiked samples were stored at temperatures of -70°C and -20°C for subsequent analyses.

Methods

LC-MS/MS method optimization

The analyte and IS were tuned on the mass spectrometer to identify their parent and daughter ions for accurate quantification with the desired response. The mass parameters were optimized in negative ionization mode, yielding mass transitions of 307.400 → 262.400 for the analyte (Figure 1) and 313.900 → 270.000 for the IS. The finalized mass spectrometric parameters are Ion spray voltage (IS) 5500, Temperature (°C)-500, Curtain gas (CUR)-40, Collision gas (CAD)-5, Gas 1-45, Gas 2-50, Declustering potential (DP)- 50, Collision Energy (CE)- -30.00, Collision cell Exit Potential (CXP)- -10.00, Entrance Potential (EP)- -10.00, Dwell time (in msec)-200.

Following optimization of the mass parameters, the Liquid Chromatography (LC) conditions were refined using aqueous samples to achieve well-defined peak shapes and ensure robust correlation between the LLOQ and ULOQ in terms of peak area and area ratio relative to the Internal Standard (ISTD) response (Figure 2). Various mobile phases and chromatographic columns were evaluated during this process. The optimized LC parameters are Gemini NX C18, 100X4.6 mm, 5 µ, column, Mobile phase-Isocratic-2 mM ammonium formate (pH:4.5±0.3) buffer in water: Acetonitrile (MeCN) (30:70v/v), Injection volume 10 µL, Sample cooler Temperature-5±2°C, Run Time-3.5 min, Column oven Temperature-35±2°C, Retention Time (RT) for Tafamidis and Tafamidis 13C6-1.35±0.4 min, Regression and Weighing factor-Linear regression and 1/X2.

Sample Extraction from Biological Matrix

Human plasma containing K₂EDTA was used as the biological matrix, and the analyte was spiked at a 2% concentration from the respective Calibration Curves (CCs) and Quality Control samples (QCs). A spiking volume of 2% was selected to minimize matrix dilution and solvent effects while ensuring uniform analyte distribution, it preserving the native matrix characteristics and supporting accurate and reproducible quantification. Drug extraction from biological matrices is a critical step for accurate quantification. Various extraction methods were explored, including Solid-Phase Extraction (SPE), Liquid-Liquid Extraction (LLE), and Protein Precipitation (PPT). Among these, LLE was selected as the extraction method for the current analysis.

The extraction procedure is as follows: 50 µL of the IS dilution and 200 µL of the spiked biological matrix aliquot were added to a tube. To this mixture, 200 µL of 0.1% formic acid was added

as a pre-treatment solution, followed by the addition of 2.5 mL of ethyl acetate. The mixture was vortexed for 10 min at 2500 rpm. Subsequently, the samples were flash-frozen using dry ice, and the supernatant was separated. The supernatant was then dried under a nitrogen evaporator. Once dried, the samples were reconstituted with 0.5 mL of mobile phase and injected into the LC-MS/MS system for analysis.

Method validation

The developed method to quantify TTR in human plasma was validated as per USFDA bioanalytical method validation guidance for industry.¹⁷ As part of Method Validation (MV) auto sampler carry over test, aqueous linearity, specificity, selectivity, specificity, precision and accuracy, matrix effect, recovery, stabilities such as short-term stock solution, freeze thaw, bench top, long term, auto sampler, dry extract, post extract, whole blood.

Data processing

The generating data acquired on LC-MS/MS analysed using Analyst software 1.6.3. as peak area ratio. The 1/X² regression was selected from the precision and accuracy batches and the same was used for all the data. The 1/X2 regression was selected based on the P & A batches data during the development, i.e. the regression of the CC assay values of P&A batches were selected in the analyst software with ZERO, 1/X and 1/X2. Among them 1/X2 back calculation data was with less mean difference.

RESULTS

Bioanalytical methods play a critical role in the successful development of drugs, enabling the quantification of these compounds in various biological matrices to generate pharmacokinetic and bioavailability data. LC-MS/MS is a superior technique in bioanalytical method development within the pharmaceutical industry due to its high selectivity and sensitivity. While several LC-MS/MS models are available, many offer limited sensitivity and dynamic linearity ranges, which can constrain the quantification of novel biological molecules. In the current study, the LC-MS/MS QTRAP 4500 was employed to quantify Tafamidis (TFM), with a LLOQ of 5 ng/mL and an ULOQ of 4000 ng/mL.

LC-MS/MS method development

The use of IS is crucial for ensuring precise quantification of biological molecules, as they improve extraction efficiency, ionization, chromatographic performance, and overall method validation, particularly in Bioavailability and Bioequivalence (BA/BE) analyses. Stable isotope-labelled internal standards are preferred in analytical methods to ensure consistency and accuracy. In this study, TFM-13C6 was selected as the IS due to its identical behavior to the analyte. The optimized mass spectrometric and LC parameters are provided in Tables 1 and 2. Mass ion optimization was performed in negative ion mode using

Electrospray Ionization (ESI), yielding transitions of 307.400 $\rightarrow$ 262.400 for the analyte (Figure) and 313.900 $\rightarrow$ 270.000 for the IS, with no significant deviation from the ion transitions reported in the studies by Hyun *et al.*¹⁵ and Lockwood *et al.*¹⁸

Method Validation

The Validation of TFM commenced with an Autosampler Carryover Test (ASCOT), which is a critical component of analytical procedures. ASCOT helps confirm whether any sample carryover occurs between consecutive injections. The test was conducted using both aqueous and extracted samples, starting with the injection of a blank sample, followed by the ULOQ sample, a re-injection of the same blank sample (twice), and concluding with the LLOQ sample. The results were compared to ensure that the response in the blank samples was <20% of the response in the LLOQ sample for the analyte, and <5% for the IS response in the LLOQ sample. No carryover was observed between injections (Figure).

Specificity is a crucial aspect of LC-MS/MS methods, as it ensures that both the analyte and IS are free from interference, not only from each other but also between multiple analytes. In the present study, TFM was analyzed using TFM-¹³C₆ as the IS, and specificity was assessed by injecting aqueous and extracted samples. The following samples were injected: the ULOQ sample without the IS, the IS sample alone, and the LLOQ sample with the IS. The results were then compared to evaluate the analyte and IS responses in the LLOQ sample containing the IS. The results confirmed that no interference occurred between the analyte and the IS (Figure). Aqueous dilutions of the Calibration Curves (CCs) and Quality Controls (QCs) were prepared, as detailed in the preparation section, and these dilutions were used to spike plasma samples at a 2% concentration for method validation. The

linearity of the aqueous dilutions was evaluated, and the results met the acceptance criteria, with accuracies within $\pm 15\%$ of their nominal concentrations.

When using plasma samples from multiple sources in Method Validation (MV) for measuring the intended analyte, it is essential to ensure that these samples are free from interference. The extraction procedure should be validated to confirm that it has no impact on biological matrices, including normal plasma, lipemic, or haemolytic samples. In this study, selectivity was assessed using six normal plasma samples, as well as two lipemic and two haemolytic samples. For each sample, one blank and one LLOQ sample spiked with the respective plasma were processed and injected under freshly prepared CCs. The results were compared, ensuring that the response in the blank plasma samples was interference-free, with the analyte response in the LLOQ sample being <20%, and the IS response in the LLOQ sample being < 5%. The results confirmed that there was no interference in the blank plasma samples obtained from multiple sources (Figure). As the chromatograms showed in Figure , the blank sample was clear without any interference (selectivity) and the LLOQ sample was detected at the lower concentration and the ULOQ sample was at high concentration.

Matrix Effect (ME) is a critical factor in the development of any bioanalytical method, as the biological matrix can either enhance or reduce the response of the quantifying ions after spiking. ME can alter ion responses and impact the quantification of known or unknown concentrations. To assess ME, the IS Normalized Matrix Factor (ISNMF) was evaluated using the plasma samples used for selectivity testing. The ISNMF was evaluated at the lower Quality Control (LQC) and High-Quality Control (HQC) levels, comparing post-extraction plasma samples with aqueous samples.

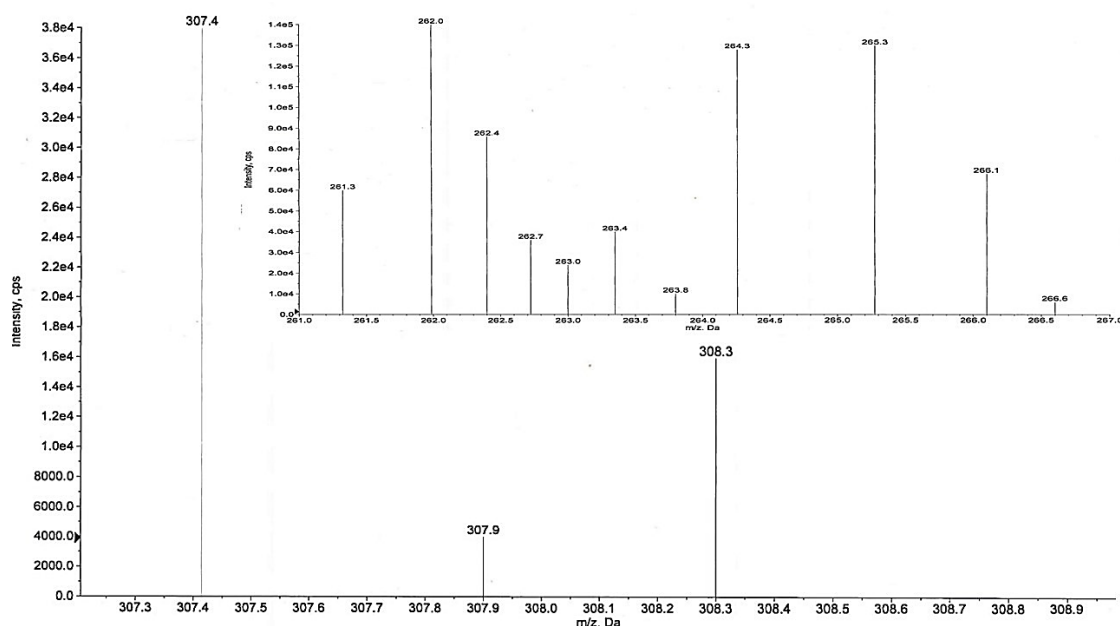


Figure 1: Q1 and Q3 Mass spectra of Tafamidis.

Table 1: Intra and Inter day Precision and Accuracy (P&A) results of Tafamidis.

		Parameter				
Parameter		LLOQ	LQC	MQC	HQC	DQC
Intraday P&A		Nominal Concentration (ng/mL)				
		5.002	15.041	1600.061	2918.754	8001.267
Intraday P&A	Mean	5.139	15.119	1536.087	3074.480	8780.812
Interday P&A	STDEV	0.3337	0.9109	73.3970	128.2432	388.3851
	%CV	6.49	6.02	4.78	4.17	4.42
	% Nominal	102.74	100.52	96.00	105.34	109.74
Interday P&A	Mean	5.168	15.250	1556.292	3067.401	8779.527
	STDEV	0.2844	0.8242	73.3970	128.2432	388.3851
	%CV	5.50	5.40	4.72	4.18	4.42

The results demonstrated that there was no matrix effect on the quantification of TFM ions due to the extraction procedure and the selected mobile phase in the developed method. The ISNMF ranged from 0.91 to 1.04 at the LQC level and from 0.99 to 1.03 at the HQC level, with a percent Coefficient of Variation (% CV) of 3.66 and 1.05, respectively. After confirming the absence of a matrix effect, the plasma lots were pooled to obtain the required amount of plasma for further analyte spiking in subsequent validation experiments.

The Precision and Accuracy (P&A) were evaluated by three P&A batches as inter and intra-day comparison. The three P&A batches were meet the acceptance criteria as per regulatory guidelines at intra and inter day batches ($n=6$). The intraday % nominal at LLOQ QC, LQC, MQC, HQC, DQC were 102.74, 100.52, 96.00, 105.34, and 109.74 with % CV 6.49, 6.02, 4.78, 4.17, and 4.42 respectively. The inter day % nominal at LLOQ QC, LQC, MQC, HQC, DQC were 103.31, 101.39, 97.26, 105.09, and 109.73 with % CV 5.50, 5.40, 4.72, 4.18, and 4.42, respectively. The results of P&A were meeting the requirements of regulatory guidelines as the % nominal at each QC level were 85-115 with the %CV less than 15. The results of developed methods on P&A evaluation shows the repeatability and validity of the analytical method to assess unknown samples (Table 1).

The developed extraction method should be validated for its extraction efficiency, how much of concentration is extractable for different steps in the procedure from known and unknown samples. The recovery of analyte and IS was evaluated at three concentration levels, i.e. LQC, MQC and HQC and the IS recovery was calculated at each level by comparing with post extraction spiking sample at each level ($n=6$). The percentage recovery from extraction procedure at LQC, MQC, HQC level was 74.19, 76.49, and 73.51 for analyte, 73.79, 75.98, and 72.31 for IS respectively. The global recovery was 74.73 for analyte and 74.03 for IS with % CV 2.09 and 2.49. The results showed that the developed extraction and analytical method showed consistent recovery at

lower to higher level of analyte in its quantification (Table 2). The recovery was approximately 75%, which is considered acceptable, the parameters evaluated in method validation supports extraction efficiency and the extraction procedure is consistent and reproducible. The method demonstrated acceptable accuracy and precision across different concentration levels, including at the lower limit of quantification. Furthermore, the sensitivity and selectivity of the validation show accurate quantification even when only a portion of the analyte is recovered.

The stability studies for developed method are very crucial in the analyte estimation at different steps such as samples preparation, extraction procedure and at storage conditions. The stabilities were conducted for stock solutions, dilutions and stability in Whole Blood (WBS), for matrix spiking samples as Freeze Thaw Stability (FTS), Long Term stability in Matrix at -20°C and -70°C (LTM), Bench-Top Stability (BTS) and stabilities after extraction procedure as dry extract stability (DES), Wet Extract Stability (WES), Autosampler Stability (ASS) and Re-Injection Reproducibility stability of samples (RIRP) at LQC and HQC levels. All the stabilities were compared with freshly spiked QC samples ($n=6$) under freshly prepared calibration curve. The acceptance criteria for each QC samples' nominal concentration should be ± 15 and % CV should be less than 15.

The stock solutions and their dilutions used in MV has been proved for their stability as Short-Term Stability (STSS, STWS) and Long-Term Stability (LTWS, LTSS). The stock solutions stability was carried at MQC prepared from stock solution and stock dilutions stabilities were carried at ULOQ and LLOQ dilution level. The STWS % stability of stock dilutions is 103.03% at ULOQ, 98.48% at LLOQ level upto 18 hr 54 min for analyte, 100.24% for IS upto 18 hr 46 min. The STSS % stability of analyte is 99.72% up to 19 hr 06 min and 97.47% for IS up to 19 hr 02 min. The LTWS % stability of stock dilutions is 98.07% at ULOQ, 99.75% at LLOQ level up to 8 days 16 min for analyte, 101.29% for

IS up to 8 days 23 min. The LTSS % stability of analyte is 99.39% up to 8 days 37 min and 98.97% for IS up to 8 days 30 min.

The FTS stabilities will provide stability data of analyte when repeatedly thawing and storage of samples at the time of analysis during unknown samples. The FTS was carried after VI cycles of retrieval and storage of samples at -70°C. During the FT cycles i.e. gap between each FT cycle the temperature of the deep freezer was monitored physically and observed there was no major alterations (i.e. $\pm 10^\circ\text{C}$). The samples showed good % stability with 103.62%, 103.06% at LQC, HQC levels. The LTS will provide stability of analyte from stating collection to last sample analysis condition

at desired storage of unknown samples and it was evaluated at -20°C, -70°C. The % stability of analyte was 102.03%, and 102.76% at -20°C for LQC, HQC level and 99.7%, and 100.30% at -70°C for LQC, HQC levels respectively. The BTS provides the stability of analyte at thawing condition at Room Temperature (RT) up to samples processing time for analysis of unknown samples. The BTS of analyte proved up to 9 hr 23 min at RT with % stability 99.26, and 100.71 at LQC, HQC level.

The ASS provides, stability of analyte in an auto sampler at storage temperature in samples after extraction, completion of an analytical run either known and unknown samples. The ASS of

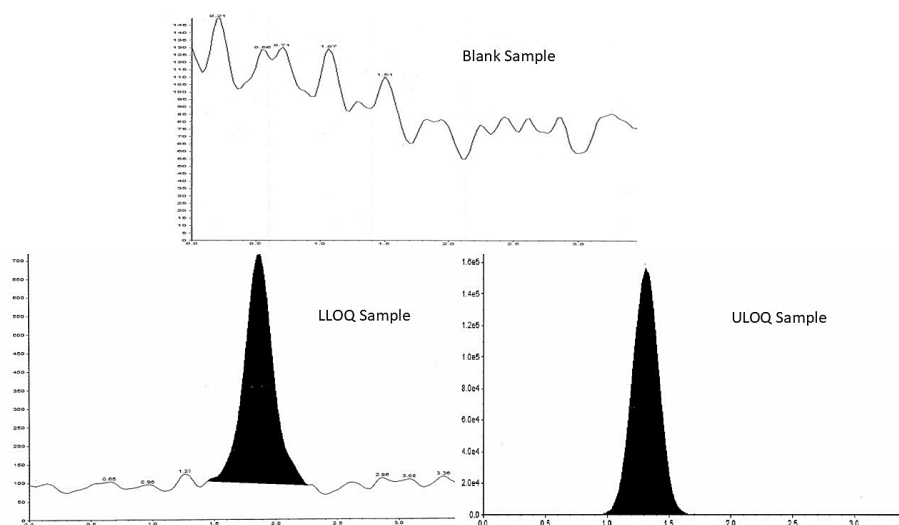


Figure 2: The chromatograms of extracted Blank, LLOQ and ULOQ samples.

Table 2: Recovery of TFM and TFM 13C6 spiked plasma samples.

Parameter for Calculation					
Parameter for Calculation	Name of QC Sample	Analyte	IS	Analyte	IS
		Response in Post spiked samples		Response in Extracted samples	
STDEV	LQC	12416.167	283079.667	9211.500	208886.167
%CV		428.196	4456.784	191.032	8609.919
% Recovery		3.449	1.574	2.074	4.122
Mean	MQC			74.19	73.79
STDEV		1278713.167	281357.000	978063.667	213780.000
%CV		21635.593	6824.906	9560.623	5802.921
% Recovery	HQC	1.692	2.426	0.978	2.714
Mean				76.49	75.98
STDEV		2458258.167	293320.500	1806945.167	212104.500
%CV	Global Recovery	48511.978	7918.038	25260.195	4247.059
% Recovery		1.973	2.699	1.398	2.002
STDEV				73.51	72.31
%CV				74.73	74.03
				1.563	1.847

analyte was 35 hr 6 min at $5 \pm 3^\circ\text{C}$ with %stability 101.09, 100.07 at LQC, HQC level. The DES and WES were stability of analyte after the extraction procedure and before going to start analysis in the instrument. The DE was stability of analyte after evaporation step and before reconstitution of sample. The analyte was stable in DE samples with % stability 99.68, and 98.61 at LQC, HQC level at $2-8^\circ\text{C}$. The WES provide stability of analyte in samples after the reconstitution and storage of them at desired temperature ($2-8^\circ\text{C}$) and % stability of analyte was 100.53, and 100.29 at LQC, HQC samples.

The stability of analyte was evaluated in Whole Blood (WB) by comparing the freshly spiking in WB and spiked WB kept for stability at $2-8^\circ\text{C}$ and analyte was stable in whole blood up to 3 hr 17 min with % stability 94.25, and 96.15 at LQC, HQC level. The RIRP was evaluated by injecting the already acquired samples (LQC and HQC) from accepted P&A batch and the re-injected samples were processed with previous CC. The re-injected samples were showed stability up to 33 hr 56 min with % stability 98.30, and 98.06 at LQC, HQC levels. The RIRP provides stability of precision of replicate injections, reproducibility of results in-case of any instrument interruptions and failures.

The analyte and IS demonstrated stability across various conditions, including short-term, long-term, freeze-thaw, bench-top, auto-sampler, dry and wet extract, whole blood, and re-injection reproducibility. Stability was maintained across different temperatures and durations, ensuring reliability and integrity in analytical results throughout the method validation.

DISCUSSION

The developed LC-MS/MS method using the QTRAP 4500 platform demonstrated high selectivity, sensitivity, and robustness for the quantification of Tafamidis (TFM) in human plasma, meeting all regulatory validation requirements. The method achieved a Lower Limit of Quantification (LLOQ) of 5 ng/mL and an upper limit of 4000 ng/mL, offering a wide dynamic range suitable for pharmacokinetic and bioavailability studies compared to previous reported methods.¹²⁻¹⁶ The use of TFM-13C₆, a stable isotope-labeled internal standard, ensured minimal variability in ionization and extraction efficiency, aligning with current best practices for bioanalytical method development and supported by recent findings in isotope-labelled analytical methodologies.¹²

Selectivity and specificity assessments across various matrices including normal, lipemic, and haemolytic plasma showed no interference at the retention times of the analyte or internal standard. This is critical for clinical sample analysis and aligns with previous approaches used in tafamidis quantification studies.¹⁴ The method demonstrated excellent linearity across the calibration range, with all calibration and Quality Control (QC) samples meeting the acceptance criteria of $\pm 15\%$ deviation from nominal values. No autosampler carryover was observed,

confirming the robustness of the injection sequence and analytical integrity during batch runs.

Matrix Effect (ME), a common challenge in LC-MS/MS bioanalysis, was rigorously assessed using the Internal Standard-Normalized Matrix Factor (ISNMF). The ISNMF ranged from 0.91 to 1.04 at the LQC level and 0.99 to 1.03 at the HQC level, with %CV values below 5%, confirming that ion suppression or enhancement was negligible. This stability across matrix conditions ensures reliable quantification of TFM in complex biological samples, supporting the method's broad applicability. Precision and accuracy evaluations yielded %CV values below 6.5% for both intra- and inter-day analyses, and % nominal values remained within 85-115% across all QC levels, confirming the method's repeatability and compliance with regulatory standards.

Extraction efficiency was consistent across LQC, MQC, and HQC levels, with mean analyte recoveries between 73.51% and 76.49%, and global recovery of 74.73%, indicating effective sample preparation. These values were reproducible with low variability, confirming the robustness of the extraction protocol. Stability studies under multiple conditions, including freeze-thaw, bench-top, autosampler, whole blood, and re-injection scenarios demonstrated that TFM and its internal standard remained stable under all tested conditions, with recoveries consistently above 94%. Long-term and short-term stability of stock solutions and dilutions also met acceptance criteria, ensuring method reliability during extended analytical runs.

Overall, the developed method offers a reliable and validated approach for the quantification of tafamidis in plasma. Its strong performance across all validation parameters including specificity, matrix effect, precision, accuracy, extraction recovery, and stability makes it suitable for clinical pharmacokinetic studies, therapeutic monitoring, and bioequivalence assessments. Compared to previously published methods, the current approach provides improved sensitivity and analytical robustness, contributing meaningfully to ongoing research in tafamidis pharmacology and clinical drug development.

CONCLUSION

The bioanalytical method developed for the estimation of tafamidis in human plasma was successfully validated in accordance with regulatory guidelines. This method demonstrated its effectiveness by achieving a LLOQ of 5 ng/mL, facilitating the accurate quantification of low analyte concentrations. Previous methods for TFM quantification in various biological matrices were associated with several limitations, such as lower recovery, longer runtimes, and reliance on high-performance Liquid Chromatography (HPLC). Moreover, some LC-MS/MS methods used structurally similar compounds as internal standards and had limited linearity ranges. In contrast, the current method marks a significant advancement. Developed using LC-MS/MS,

it incorporates a deuterated internal standard, which enhances both accuracy and reproducibility. Unlike HPLC methods, which rely on calibration curves and compound absorbance for quantification, LC-MS/MS utilizes ionization and mass-to-charge ratios, enabling the detection and quantification of analytes at much lower concentrations. This method also employs an extraction procedure with higher recovery, minimal Matrix Effects (ME), and excellent reproducibility. Its robustness makes it well-suited for clinical studies on TFM, structurally related bioactive molecules, novel compounds, and new formulations. Additionally, it can be applied to various biological matrices in bioavailability and bioequivalence studies.

ACKNOWLEDGEMENT

The authors thank VFSTR and for their support and ideas and also thank Aragen Labs for providing lab space and other assistance for the work.

ABBREVIATIONS

HPLC: High Performance Liquid Chromatography; **IS:** Internal Standard; **LC-MS/MS:** Liquid Chromatography-Mass Spectrometry/Mass Spectrometry; **LLOQ:** Lower Limit of Quantification; **mL:** Milli Liters; **ng:** Nanograms; **SD:** Standard Deviation; **TFM:** Tafamidis; **ULOQ:** Upper Limit of Quantification.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

The current research did not receive any funding.

SUMMARY

High sensitivity, accuracy, and repeatability are obtained by a validated LC-MS/MS method for TFM quantification in human plasma with LLOQ of 5 ng/mL. It exceeds previous techniques with

better recovery, minimal matrix effects, and shorter runtimes by means of a deuterated internal standard and effective extraction. Studies on clinical, bioavailability, and bio-equivalency across various biological matrices will find the approach perfect.

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Cite this article: Aitha S, Devaraju S, Tatapudi KK, Reddy GMK, Somasekhar T. Development and Validation of a Simple and Robust Bioanalytical Method to the Quantification of Tafamidis in Human Plasma using LC-MS/MS. *Indian J of Pharmaceutical Education and Research*. 2026;60(4s):s1716-s1723.